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Research Article

Evaluation of the Effect of Naringenin Liposomal Formulation on Retinopathy in an Experimental Rabbit Model

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Abstract: Neovascularization is a hallmark of diabetic retinopathy (DR). Naringenin has protective effect against DR. Liposomes are suitable Nano-carriers for ocular drug delivery. Hence, we prepare and evaluate the effect of naringenin liposomal formulation (NLF) on DR in a rabbit model. NLF was prepared by thin lipid film method. NLF characteristics were evaluated by Scanning Electron Microscope (SEM), Dynamic Light Scattering (DLS) and zeta potential. NLF release pattern and stability were assessed. Thirty-six rabbits were divided into six groups (control, placebo, bevacizumab and NFL (200, 500 and 800 µg/mL)). Intravitreal injection of Alpha-aminoadipic acid (α-AAA) induced retinopathy. NFL was administered for three weeks. Neovascularization scoring was done by an eye examination. Furthermore, histological evaluation of retina was performed for angiogenesis and dilation of vessels. SEM imaging revealed successful NLF preparation. The particle size obtained 148 to 215 nm. Encapsulation Efficiency were 43% and 66% which is good for naringenin. Zeta potential was 15mV. Two formulations of NLF showed suitable release (% drug released after 1 h (%D1) was 5.5% and 3.1% and % drug released after 24 h (%D24) was 72.93% and 52.01%). NLF exhibited an acceptable stability. Histological findings confirmed neovascularization. Treatment with bevacizumab and three doses of NLF significantly decreased neovascularization score, respectively. Furthermore, histological results revealed that three doses of NLF especially 800 µg/mL improved damage caused by α-AAA in the retina. NFL showed protective effects against neovascularization. We showed that NFL is capable of nanoformulation with a great attenuating effect against neovascularization. Finally, the findings revealed that NFL is an anti- neovascularization agent.

Keywords: *Liposome, Naringenin, Neovascularization, Rabbit, Retinopathy*

Deneysel Tavşan Modelinde Naringenin'in Lipozomal Formülasyonunun Retinopati Üzerine Etkisinin Değerlendirilmesi

Öz: Neovaskülarizasyon, diyabetik retinopatinin (DR) bir özelliğidir. Naringenin, DR'ye karşı koruyucu bir etkiye sahiptir. Lipozomlar, oküler ilaç dağıtımı için uygun nanotaşıyıcılardır. Bu nedenle, bir tavşan modelinde naringeninin lipozomal formülasyonunu (NLF) hazırladık ve DR üzerindeki etkisini değerlendirdik. NLF, ince lipid film yöntemiyle hazırlandı. NLF özellikleri, Scanning Elektron Mikroskobu (SEM), Dinamik Işık Saçılımı (DLS) ve zeta potansiyeli ile değerlendirildi. NLF salınım modeli ve stabilitesi değerlendirildi. 36 tavşan; kontrol, plasebo, bevacizumab, 200 µg/mL NFL, 500 µg/mL NFL ve 800 µg/mL NFL olmak üzere altı gruba ayrıldı. Alfa-aminoadipik asit (α-AAA)'in intravitreal enjeksiyonunu takiben retinopati oluşturuldu. Üç hafta boyunca NFL uygulandı. Göz muayenesi ile neovaskülarizasyon skorlaması yapıldı. Ayrıca anjiyogenez ve damarların dilatasyonu için retinanın histolojik değerlendirmesi yapıldı. SEM görüntüleme, NLF hazırlığının başarılı olduğunu ortaya çıkardı. Elde edilen partikül boyutu 148 ile 215 nm arasındaydı. Kapsül oluşturma verimliliği %43 ve %66 olup naringenin için oldukça iyiydi. Zeta potansiyeli 15mV idi. NLF'nin iki formülasyonu da uygun salınım gösterdi (1 saat sonra salınan ilaç yüzdesi (%D1) %5.5 ve %3.1 ve 24 saat sonra salınan ilaç yüzdesi (%D24) %72.93 ve %52.01 idi). NLF, kabul edilebilir bir kararlılık sergiledi. Histolojik bulgular neovaskülarizasyonu doğruladı. Sırasıyla bevacizumab ve üç doz NLF ile tedavi, neovaskülarizasyon skorunu önemli ölçüde azalttı. Ayrıca histolojik sonuçlar, üç doz NLF'nin özellikle de 800 µg/mL'nin retinada α-AAA'nın neden olduğu hasarı iyileştirdiğini ortaya koydu. NFL, neovaskülarizasyona karşı koruyucu etkiler göstermiştir. NFL'nin neovaskülarizasyona karşı büyük bir zayıflatıcı etki ile nanoformülasyon yapabildiğini gösterdik. Son olarak, bulgular NFL'nin bir anti-neovaskülarizasyon ajanı olduğunu ortaya koydu.

Anahtar sözcükler: *Lipozom, Naringenin, Neovaskülarizasyon, Retinopati, Tavşan*

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Introduction

Retinopathy is called any damage to the retina of the eye [1]. Diabetes [2], hypertension [3], and prematurity of infants [4] develop retinopathy. Diabetic retinopathy (DR) is a chronic complication that affects almost all diabetic patients [5]. DR is the fifth leading cause of blindness and visual disorders. Global prevalence of DR is 22.27% and 103.12 million adults live with DR worldwide in 2020 [6]. The retinal microvascular changes in DR. The permeability of the arteries increases and the blood vessels in the retina proliferate abnormally in response to the lack of blood supply [7]. Moreover, an increase in the thickness of the basement membrane of capillaries occurs in DR, which is related to the deposition of advanced glycation end products (AGEs). As the thickness of the basement membrane increases, the capillaries become blocked and the retina becomes ischemic [8]. AGEs also increase vascular endothelial growth factor (VEGF) expression in microvascular endothelial cells [9]. Retinal cells need high oxygen, so hypoxia is caused by vascular damage in the retina [10]. Hypoxia stimulate VEGF production [11]. VEGF reduces blood retinal barrier (BRB) damage and increases vascular leakage by reducing the expression of occludin, as binding proteins and protector of the BRB [12]. Thus, VEGF plays important role in neovascularization in retina [13]. Alpha-aminoadipic acid (α-AAA) is used for induction of neovascularization model similar to retinal injury in DR. α-AAA as the analog of amino acid, L-glutamic acid exhibits gliotoxic effects in the retina [14]. Hence, inhibition of VEGF production reduces capillary vascular leakage. Therefore, the use of anti-VEGF therapies could treat retinopathy [15]. Bevacizumab is a recombinant human monoclonal antibody that affects all VEGF isoforms. Bevacizumab could treat many retinopathies due to increasing VEGF activity including DR [16].

Despite the effectiveness of synthetic drugs, their side effects have raised concerns [17]. Studies have turned their attention to active compounds derived from plants [18-22]. Flavonoids, as the largest group of polyphenols, exhibits many therapeutic effects [23]. Naringenin is a flavanone exerts antioxidant, free radical scavenging, anti-inflammatory and immunomodulatory properties [24]. Naringenin could ameliorate diabetic retinopathy through the exhibition of antioxidant and anti-inflammatory effects [25]. Moreover, naringenin has an inhibitory effect against angiogenesis and VEGF production [26]. Medications used to treat eye diseases often have a short shelf life and little eye contact. One way to increase the shelf life, solubility and bioavailability of drugs is to use nanoparticles for drug delivery [27]. The use of nanoparticles for drug delivery to treat various diseases is increasing rapidly. Loading of drugs and effective plant compounds in nanoparticles has been considered due to the increase in drug shelf life [28]. Because of their biocompatibility and lack of toxicity, nanoliposomes are recommended as promising nanocarriers for ocular administration [27]. Furthermore, it has reported that liposomal nanoformulation could enhance solubility and bioavailability of naringenin and participate in controlled delivery of naringenin (a,b). Previous studies have shown the therapeutic effect of NLF against various diseases including nonalcoholic fatty liver disease (NAFLD) (c). Hence, we decided to prepare and evaluate of the therapeutic effect of naringenin liposomal formulation (NLF) on retinopathy in a rabbit model.

Material and Methods

Ethical Approval

The Animal Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS) approved all of the experimental protocols (IR.AJUMS.ABHC.REC.1399.064).

Chemicals

The following chemicals: Cholesterol (Merck Company, Germany), Lecithin (Merck Company, Germany), Naringenin (Merck Company, Germany), DL-Alpha-aminoadipic acid (Sigma Company, Germany), Stearylamine (Gattefossé Company, France), Ethanol (Merck Company, Germany), Methanol (Samchun Company, Korea), Chloroform (Samchun Company, Korea) were used in the current study.

Preparation of Liposome

The liposome nanoparticles were prepared by the thin-film hydration method. Briefly, phospholipids and drugs including lecithin, cholesterol, stearylamine and naringenin were dissolved in 50 mL of organic solvent containing chloroform and methanol (2:1) and the chloroform and methanol solution was evaporated by Rotary Evaporator (120 rpm) at 60°C to prepare thin lipid film. The sample was vacuumed overnight to remove the remaining solvent. In the next step, the volumetric flask was heated to 45°C and after adding the aqueous phase including the phosphate buffer solution, the mixture was vortexed by hand and stirred for 20 min.

Liposome Physicochemical Characterization

Liposome Size: Particle size was measured at 25°C by particle size analyzer. The mean droplet size of samples was determined by SCATTER SCOPE 1 QUIDIX (South Korea) based on photon correlation spectroscopy with a wide measurable size range (1-7000 nm). Each sample was measured triplicate.

Zeta Potential: Zeta Analyzer was used to check the surface charge. The Zeta Potential of samples was determined using a Malvern Zetasizer Nano-range instrument (Malvern Instruments Ltd., Malvern, UK).

Morphologic Characterization

Scanning Electron Microscopy (SEM): To perform the SEM imaging, the shape and structure of dried samples of the liposome was examined at 15 kV with a 6300 field emission scanning electron microscope (Hitachi, S-4160).

Naringenin Loaded Liposome Calculation

For this purpose, liposomal formulations were centrifuged at 12.000 rpm for 30 min. Then we separated the liposomes from the surface of the solution and centrifuged the aqueous solution containing the unloaded drug again, and this time we separated the aqueous solution and the liposomes again for 15 min. To calculate the amount of unloaded drug, the underlying liquid was isolated and the amount of drug was measured. The amount of loaded drug was calculated by differentiating the amount of unloaded drug from the total drug used. On the other hand, to confirm the test results, the upper liposomal phase was dissolved in methanol to break the liposomes. Then the amount of loaded drug was calculated. In this way, both loaded and unloaded drugs were calculated and the percentage of loaded drugs was calculated based on the following equation:

Entrapment efficiency = Trapped drug/Total drug x100

Naringenin Loaded Liposome Release Pattern

Static diffusion cells were used for this purpose. After separating the liquid phase and the liposomal phase after centrifugation, 2 mL of the liposomes were separated and placed in the donor phase. The amount of drug passing through the cellulose membrane that enters the acceptor phase, which is the artificial tear environment of the eye with a pH of 7.4, was then measured for 24 h. By plotting the cumulative amount of drug passed over time, the pattern of drug release from the liposome was determined.

Liposome Stability Evaluation

In order to evaluate the stability of liposomal formulations, the samples were kept at room temperature for three months. After this period, the formulations were evaluated for drug loading capacity, suspension stability and particle size. Decreased loading capacity or cake formation in the suspension and particle size change are considered as signs of instability.

Naringenin Amount Determination

The amount of drug was determined by HPLC with c18 column at a wavelength of 288 nm.

pH Test

pH was measured by a digital pH meter [29].

Animals and Study Design

The experimental procedures were conducted at Animal Laboratory, affiliated to Lorestan University of Medical Sciences, Khorramabad, Iran. A total of thirty-six adult male albino New Zealand rabbits weighing 1.5 to 2 kg were chosen for this investigation based on previous studies and kept in standard cages (d). The rabbits were housed under appropriate environmental conditions that included a temperature of 22°C, 12 h light-dark cycles, and with free access to standard laboratory food and tap water.

Induction of Retinopathy: By injecting alpha-amino adipic acid, which is an angiogenic agent, into adult male New Zealand albino rabbits, we caused angiogenesis and retinopathy, and the retinopathy caused by alpha amino adipic acid injection was similar to diabetic retinopathy. Eight weeks after the injection of the mentioned compound, the occurrence of retinopathy was confirmed by the ophthalmologist using clinical examination and microscopic images. The animals' health status and detailed eye examination was done before the study by a physician. Intraocular injection of Alpha-Aminoadipic acid (α-AAA) was used to induce retinopathy. Fifty μL of 0.025 M α-AAA was injected into a rabbit's eye using a 27-gauge (27 μm) needle. Lidocaine eye drops alone were used for anesthesia before injection and tetracycline eye drops was used to prevent infection after injection of Alpha-Aminoadipic acid. A 27-gauge needle was inserted into the vitreous cavity, 1.5 mm posterior to the superotemporal limbus, and the needle tip was directed into the midvitreous under direct visualization with external illumination of indirect ophthalmoscopy. Ophthalmoscope was used to evaluate the retina for the development of retinopathy.

Grouping: The rabbits were randomly divided into the following six groups (six rabbits per group):

Group 1: NLF treated group with a concentration of 200 μg/mL

Group 2: NLF treated group with a concentration of 500 μg/mL

Group 3: NLF treated group with a concentration of 800 μg/mL

Group 4: Selected placebo formulation treated group (received vehicle = liposomal formulation without naringenin)

Group 5: Bevacizumab treated group with intravitreal injection of a concentration of 1.25 mg/50 μL using a 27-gauge needle

Group 6: Healthy non-diabetic animals.

Two months after induction of retinopathy, the treatment continued by administration of drops of different concentrations of the selected liposomal formulation twice daily for 5 days a week for three weeks. The administration of drops was carried out in the right eye and the left eye was considered as negative control (untreated retinopathy).

Neovascularization Scoring: After induction of retinopathy on the 8th weeks following injection of α-AAA, neovascularization evaluation was performed using ophthalmoscopic examination by a physician according to the scoring between zero and one as follow:

Index1: Complete normal retinal neovascularization to the end of zone III (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis)

Index2: Change the color of the edges from pink to white (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis)

Index3: No increase in disease severity (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis)

Index4: Blood vessels cross from the line demarcation border (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis)

Index5: Start the process of replacing active lesions with zero-grade scar tissue (Zero=The lowest amount of angiogenesis and 1= The highest amount of angiogenesis) [15,30].

It is necessary to mention that mydriatics (Tropicamide 1% w/v, eye drops) (MYDRAX®, Sinadarou Labs Co., Iran) used prior to eye examinations for neovascularization scoring.

Histological Evaluation

To conduct histological evaluation, 0.5 mL of 2% sodium fluorescein solution was injected intraperitoneally, and then the rabbits were euthanized with sodium phenobarbital at a dose of 200 mg/kg. In histological evaluation, the aim was to detect neovascularization and observe dilated vessels. For this purpose, the removed eye was placed in a mixture of 8% formaldehyde, 30% ethanol and 10% glacial acetic acid for 24 h at 4°C and then transferred to a 70% ethanol solution. Next, tissue was embedded in paraffin and cut into a 5 µm thick sections. Finally, the sections were stained with hematoxylin and eosin (H&E) and tissue changes and neovascularization were examined in all samples [31].

Statistical Analysis

Data analysis was carried out using SPSS 24 analytic software (SPSS, Inc., Chicago) and Graph Pad Prism (Version 8.01). Statistical analysis was performed on the effect of independent variables on physicochemical properties of formulations (Particle Size, Naringenin amount and pH parameters) by one-way ANOVA using Minitab software. Kruskal-Wallis non-parametric statistical test was used for neovascularization parameter and Mann-Whitney was used for pairwise comparison. All data were displayed as mean ± standard deviation (SD). Differences with p-value ≤0.05 were considered significant.

Results

Liposome Characterization

Different liposome formulation's components are based on factorial design and formulation characters including the Particle size and Encapsulation Efficiency (EE%) demonstrated in *Table 1*. Through this experiment, particle sizes were between 148 to 215 nm. Low particle size provided high surface area and so high drug partitioning into the eye. Also, EE% values were between 43% and 66% so it can be considered a good value for a lipophilic compound such as naringenin. Due to the need for a particle size below 200 nm for the appropriate ocular formulation, formulations No. 5 and 7 were selected for release studies and the selection of the final formulation.

Zeta Potential

As shown in *Fig. 1*, Zeta potential was obtained as 15 mV for NLF.

Morphology of NLF

As displayed in *Fig. 2*, the SEM image showed vesicular structures with an average diameter of 150 nm, that is, the following results reported by the particle size analyzer in this study.

Release Study

The percentage of released drugs is an important characteristic of the formulation which plays an important role in formulation effectiveness. Drug release profiles

Table 1. *Liposomal formulation's components and liposome characterization*

Formulations	%Loading (EE %)	PZ	Factorial	Aqueous Volume	%Lipid	Drug	Blocks	PtType	RunOrder	StdOrder
1	55.0±2.65	204±14.4	NNP	10	5	0.15	3	1	17.15.1	13.11.9
2	46.0±4.36	207±17.9	NPN	10	10	0.1	3	1	19.9.2	5.13.21
3	51.0±4.00	214±15.6	NNN	10	5	0.1	3	1	22.13.3	1.9.17
4	55.3±9.02	202±6.00	PNP	15	5	0.15	3	1	26.14.4	4.12.20
5	66.0±2.65	147±21.4	NPP	10	10	0.15	3	1	24.11.5	7.15.23
6	51.0±6.56	206±8.15	PPN	15	10	0.1	3	1	21.12.6	6.14.22
7	64.0±6.00	168±6.51	PPP	15	10	0.15	3	1	20.16.7	8.16.24
8	43.0±2.65	215±19.55		15	5	0.1	3	1	18.10. 8	2.10.18

EE: Encapsulation efficiency, PZ: particle size; N0. 1-8 show liposomal formulations; No. 5 and 7 were selected finally because of their particle size (<200 nm)

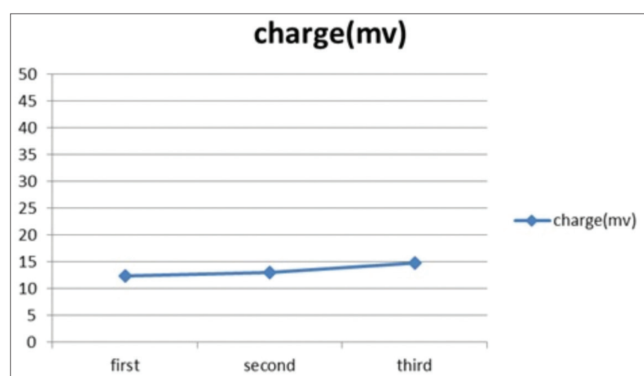


Fig 1. Zeta potential of NLF

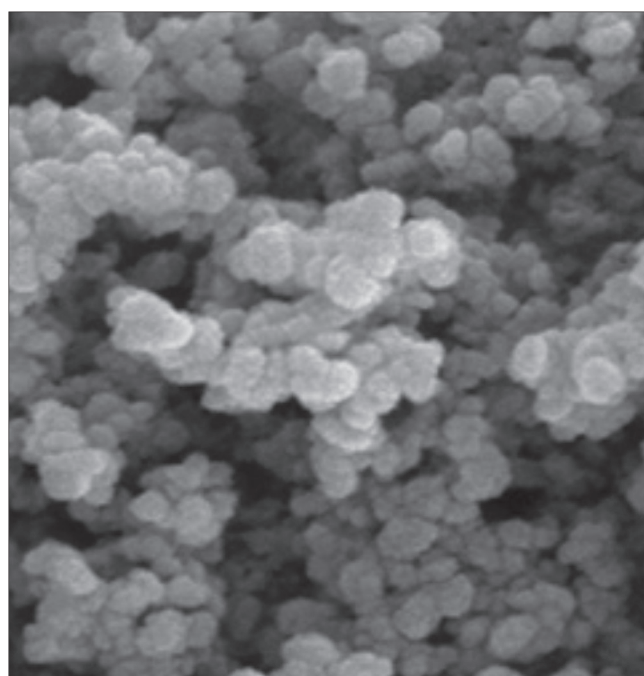


Fig 2. Scanning electron microscopy (SEM) image of liposomal formulation No.5 (as selected formulation) represents particle size as 147 nm

Table 2. Percentage of naringenine release after 1 h (%D1) and 24 h (%D24) in d liposomal formulations

Liposomal Formulations	Percentage of Naringenine Release	
	%D1 (mean±SD)	%D24 (mean±SD)
No. 5	6.67±0.42	77.3±6.13
No. 7	7.33±0.23	53±3.26

Naringenin release through liposomal formulations NO.5, 7

for each formulation were provided in buffer phosphate. The percentage of drug released after 1 h (%D1) and 24 h (%D24) as a sign of fast and sustained release, respectively, are shown in *Table 2*.

It was observed that %D1 for batch No. 5 was 5.5% and batch No. 7 was 3.1%. Also, %D24 values were 72.93% and 52.01% belongs to batch No. 5 and 7.

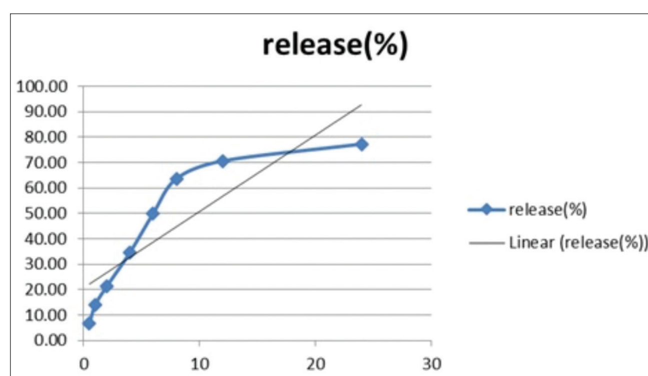


Fig 3. Percentage of naringenine release in liposomal formulation No. 5.

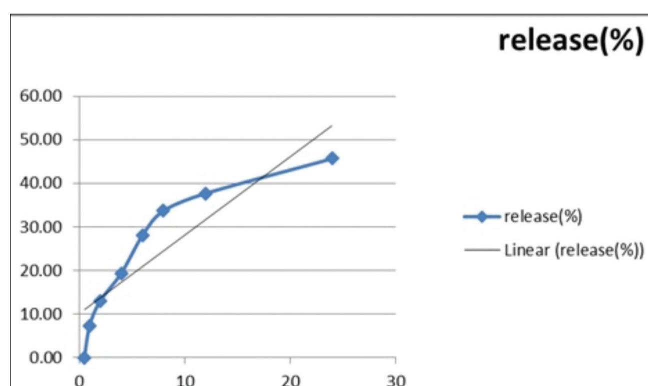


Fig 4. Percentage of naringenine release in liposomal formulation No. 7

The release profiles of naringenin through selected liposomes are illustrated in *Fig. 3, Fig. 4*.

Formulation No. 5 was selected for clinical studies at the specified concentrations due to the cumulative release rate as well as the higher loading rate.

Stability of Selected Liposomes

The stability of naringenin-loaded optimized liposome was assessed by checking the change in EE%, particle size, and cake formation of optimized liposomes stored at room temperature for 3 months. Results revealed no cake formation and change in EE%. The change in particle size for 3 months is illustrated in *Fig. 5*.

As shown in *Table 3*, no significant difference was seen in the average particle size of the optimized liposomes for 3 months after storage at room temperature (P=0.979).

Naringenin Amount Determination

The amount of naringenin in liposomal formulation has been shown in *Table 4*. Significant difference was seen in the amount of naringenin in liposomal formulation between different concentrations (P<0.001).

The pH of NLF has been shown in *Table 5*. No significant difference was observed in the pH of naringenin liposomal formulation between different concentrations (P=0.765).

Neovascularization Scoring

As shown in *Table 6* and *Fig. 6*, clinical scoring of neovascularization reached to peak significantly four and especially eight weeks after injection of α-AAA (P<0.001). It is necessary to mention that injection of α-AAA did not show any complications *(Fig. 7)*. On the other hand, treatment with NLF at the doses of 200, 500 and 800 μg/mL could significantly decrease clinical score of neovascularization compared to the treated group with placebo (P<0.001). This means that significant reducing clinical scoring of neovascularization was associated with increasing the duration of treatment (1, 2 and 3 weeks) and increasing the dose of NLF (200, 500 and 800 μg/mL).

Histological Findings

As shown in *Fig. 8*, prepared sections showed that the eyes of the placebo rabbits have numerous and dilated blood vessels and many erythrocytes in the retinal stroma. This indicated the occurrence of extensive angiogenesis in the retina compared to control group [31]. In the bevacizumab group, the retinal stroma had a limited number of vessels and was very similar to the control group. It was also seen that the number of blood vessels reduced in the NLF treated group with a concentration of 200 μg/mL, but a number of dilated vessels were still observed. Furthermore, the number of blood vessels also reduced in the NLF treated group with a concentration of 500 μg/mL and a much smaller number of dilated blood vessels were seen. In the NLF treated group with a concentration of 800 μg/mL, the microscopic structure showed a high improvement and was very similar to the bevacizumab and control groups.

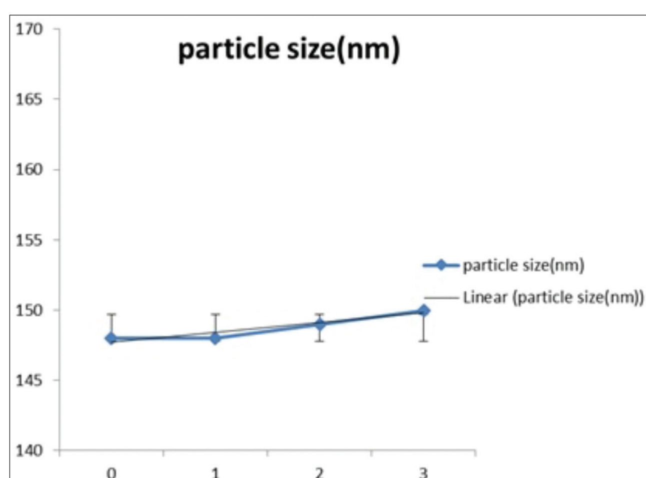


Fig 5. The changes in particle size during 3 months in selected liposomal formulation (No. 5)

Discussion

Nanoparticles are suggested for drug delivery in wide range of diseases including ocular diseases. The selection of a suitable nanocarriers with appropriate drug loading rate, slow release, high shelf life and low toxicity has a great importance for drug delivery. Nanoliposomes are one of the most widely used nanocarriers used in clinical applications for drug delivery in various diseases. Liposomes are conventional nanocarriers utilized to ocular drug delivery. Liposomes displayed several benefits such as the ability to elevate the concentration of drug in ocular tissues, enhancing drug penetration, controlling drug release, lack of toxicity or irritation to the eyes [32,33]. Hence, we decide to prepare, and evaluate of the therapeutic effect of NLF on retinopathy in animal model.

Table 3. *The changes in particle size during 3 months in selected liposomal formulation (No. 5)*

Parameter	Time of Stability				P-value
	1st Day	1st Month	2nd Month	3rd Month	
Particle size (nm)	148±2.00	148±8.72	149±6.24	150±7.81	0.979

Table 4. *The amount of naringenin in liposomal formulation*

Parameter	Concentration			P-value
	200 μg/mL	500 μg/mL	800 μg/mL	
Naringenin amount	0.1811±0.0229[c]	0.37494±0.00882[b]	0.4898±0.0497[a]	<0.001

[a-c] *Dissimilar letters show a significant difference*

Table 5. *The pH Evaluation of Selected Liposomal Formulation (No.5)*

Parameter	Concentration			P-value
	200 μg/mL	500 μg/mL	800 μg/mL	
pH	6.87±0.252	6.9±0.1	6.8±0.1	0.765

Table 6. Clinical scoring of neovascularization based on the time course of treatment in different treated groups

Intervention	Group						P-value
	Control	Treated with Bevacizumab	Treated with Placebo	Treated with NLF 200 µg/mL	Treated with NLF 500 µg/mL	Treated with NLF 800 µg/mL	
Before injection of α-AAA	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	1.000
Four weeks after injection of α-AAA	0.00±0.000[b]	3.91±0.12[a]	3.67±0.18[a]	3.88±0.21[a]	3.83±0.16[a]	3.66±0.3[a]	<0.001
Eight weeks after injection of α-AAA	0.00±0.000[b]	4.86±0.09[a]	4.86±0.15[a]	4.8±0.1[a]	4.9±0.14[a]	4.83±0.02[a]	<0.001
One week after treatment with NLF	0.00±0.000[e]	1.1±0.2[d]	4.9±0.2[a]	3.4±0.20[b]	3.2±0.1[b]	1.8±0.1[c]	<0.001
Two weeks after treatment with NLF	0.00±0.000[c]	0.8±0.1[bc]	5.00±0.2[a]	2.7±0.2[b]	1.8±0.2[bc]	1.2±0.1[bc]	<0.001

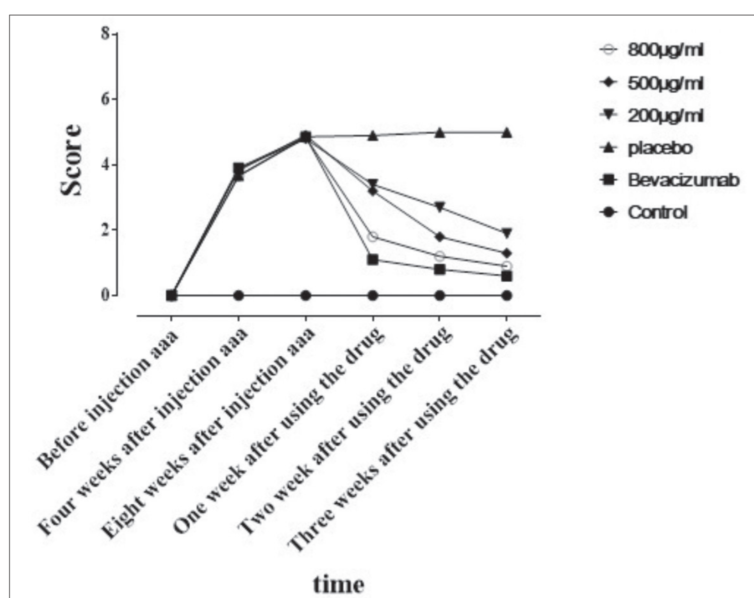


Fig 6. Clinical scoring of neovascularization based on the time course of treatment in different treated groups. Symbols shows clinical scoring of neovascularization in different groups before and after tretment

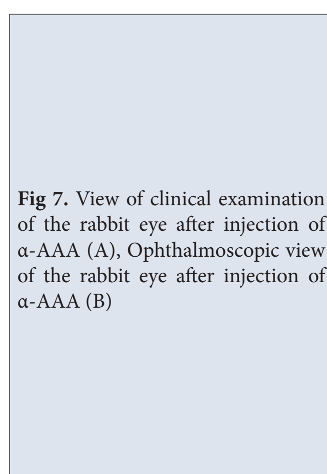
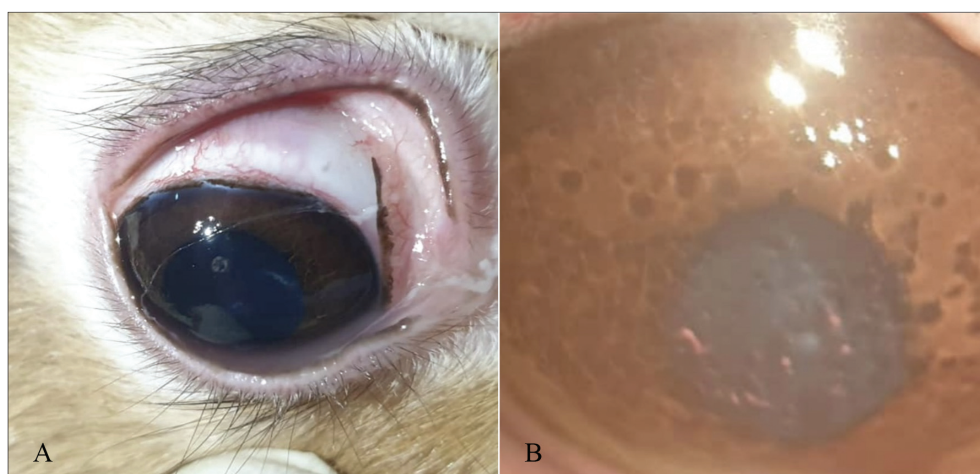


Fig 7. View of clinical examination of the rabbit eye after injection of α-AAA (A), Ophthalmoscopic view of the rabbit eye after injection of α-AAA (B)



The results of our study revealed that particle sizes of NFL were obtained between 148 to 215 nm. Moreover, the SEM image showed vesicular structures with an average diameter of 150 nm. Particle size is one of the most important absorption parameters. Smaller particles can provide more surface area for absorption. Reducing the particle size to the nanometer scale, especially below 100 nm, increases the desired properties such as stability and transparency of the system. The change in particle size occurs due to the phenomena of aggregation

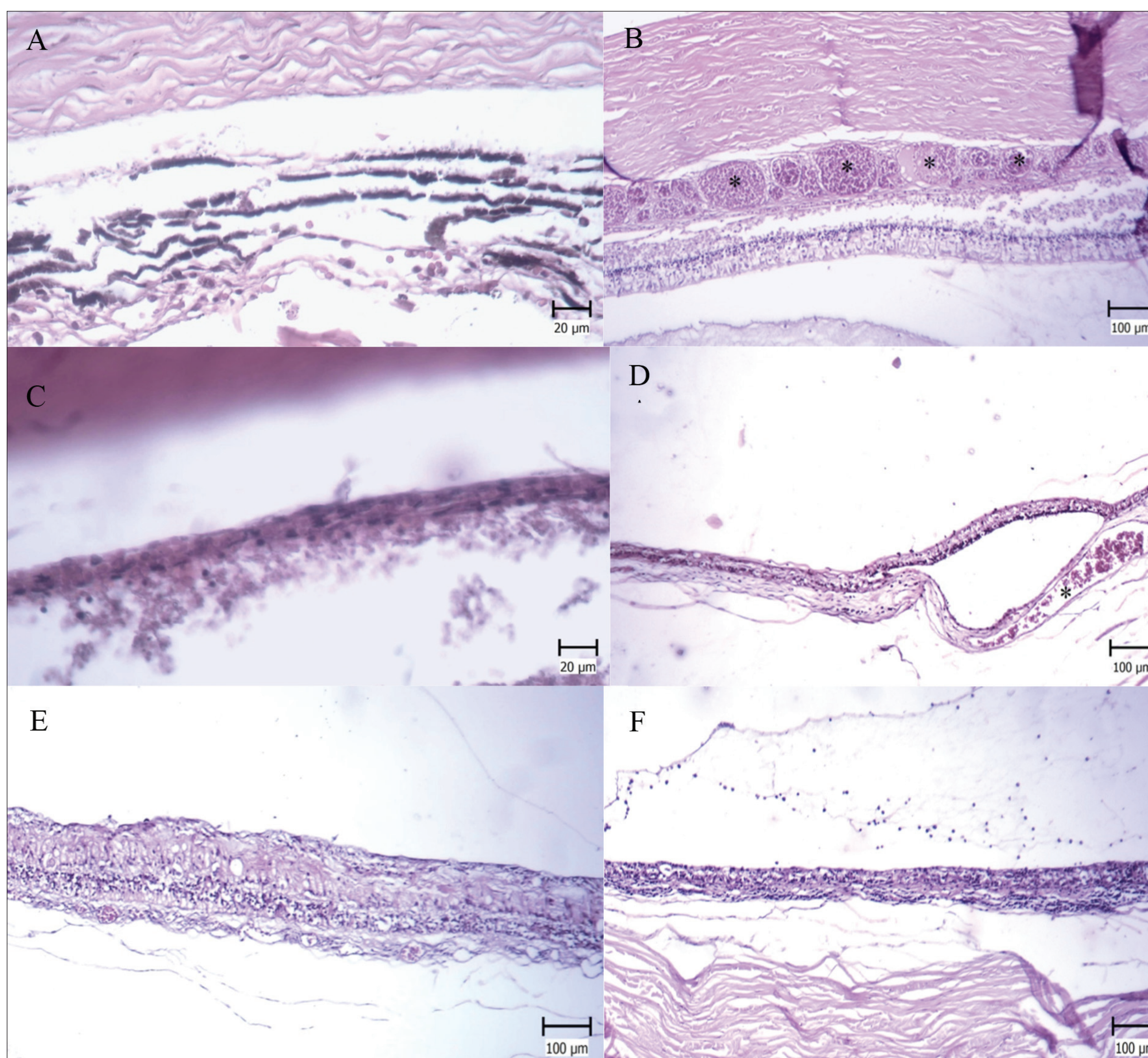


Fig 8. Histological findings of neovascularization. Control group (A), Placebo group (B), Bevacizumab (C), NLF 200 μg/mL (D), NLF 500 μg/mL (E) and NLF 800 μg/mL (F). Placebo group showed numerous and dilated blood vessels and many erythrocytes in the retinal stroma. Bevacizumab and NLF 800 μg/mL showed retinal stroma similar to control group. X40, H&E

and miscibility [34]. Basically, lecithin acts as the main skeleton in the formulation of liposomes and cholesterol is added to the formulation to increase the stability and stabilization of the liposomes. Thus, lecithin-rich liposomal membranes and the arrangement of acyl chains in one direction reduce the spaces created by the large polar groups in the lipid head, which in turn increases the contact and interaction between the chains. Cholesterol, on the other hand, may reduce the binding of hydrophobic molecules in bilayer membranes [35]. There have been various reports on the effect of cholesterol on liposome particle size, in which the liposome preparation method seems to have been very effective. The formation of complex colloids is accompanied by an increase in the particle size distribution. According to research, changes in physical properties such as particle size and particle size distribution of this liposomal formulation may be due to the accumulation of liposomes [34]. Malheiros et al.[36] concluded that the addition of cholesterol increased the diameter of vesicles in liposomes containing nisin. But, Gopinath et al.[37] reported that the increase in cholesterol concentration did not cause a significant change in particle size.

We showed that EE% values were between 43% and 66% so it can be considered a good value for a lipophilic compound such as naringenin. High EE% is recognized as an important factor utilized to state the liposomal formulation quality. Wang et al.[38] understood that Naringenin solubility and bioavailability could increase via Liposomal Nanoformulation. They obtained EE% of this formulation as 72.2±0.8%. The results of their study were consistent with our study.

Zeta potential was obtained as 15 mV for NLF. Zeta

potential is an important physical property of liposomes that is an important factor in determining the stability of colloidal systems and is the best indicator for determining the surface electrical status of dispersions. This factor indicates the amount of charge accumulation in the immobile layer and the intensity of adsorption of opposing ions to the particle surface, resulting in electrostatic stability. Zeta potential values of about 25 V m (positive or negative) are considered a criterion for separating the surfaces of particles with high and low electric charge. Colloidal systems containing particles with low zeta potential (positive or negative) have a high tendency to accumulate in the absence of other inhibitory factors such as high viscosity and spatial inhibition [39]. Cholesterol is a neutral molecule and the negative charge of the particles can be caused by the formation of a hydrogen bond between the choline group in phosphatidylcholine and the hydroxyl group in the cholesterol head. As a result of the formation of this bond, the positively charged choline group is drawn into the membrane and the negatively charged phosphatidyl group is drawn to the membrane surface, thus increasing the negative charge of the particles and the electrostatic repulsion between them [40]. Malheiros et al.[36] showed that the addition of cholesterol to the niacin-containing liposome increased the zeta potential.

It was observed that %D1 for batch No. 5 was 5.5% and batch No. 7 was 3.1%. Also, %D24 values were 72.93% and 52.01% belongs to batch No. 5 (selected formulation) and 7. Results revealed no cake formation and change in EE%. Moreover, no significant difference was seen in the average particle size of the optimized liposomes for 3 months after storage at room temperature. Release of drug from nanoliposomes depends on various factors including composition, synthesis method, type of drug and environmental conditions (temperature and pH). In the study of Tohidlou et al.[41] which used thin lipid film for preparation of liposomes, the %D1 was 5.8% and %D24 was 30%.

Another part of our result showed that clinical scoring of neovascularization enhanced significantly after injection of α-AAA. In addition, treatment with bevacizumab and NLF at all doses significantly decreased clinical score of neovascularization.

The rabbit model of neovascularization is recognized as a typical model for preclinical studies comparing the anti-angiogenic effects of various compounds. Emerging data have revealed that Bevacizumab could prevent neovascularization as an effective anti-angiogenic agent. It showed that bevacizumab-loaded albumin nano-particles played role in the treatment of corneal neo-vascularization. The results of their study exhibited that bevacizumab-loaded albumin nanoparticles could decrease fibrosis, inflammation and edema in the rat model of neovascularization.

Naringenin is a flavonoid which has been shown protective against retinopathy in several studies [42,43]. Al-Dosari et al.[44] exhibited that naringenin could attenuate oxidative stress and apoptosis in diabetic retinopathy. In other studies, it has been determined that the administration of naringenin and other natural compounds in new drug delivery system [45,46] in the form of eye drops helps to inhibit corneal neovascularization through its anti-inflammatory and antioxidant properties [47,48]. Because effective antioxidant compounds [49] have antimicrobial, anti-inflammatory and restorative properties [50-52]. Naringenin has a low solubility which may lead to limitation in its bioavailability [38]. Researchers have been recommended liposomal nanoformulations for increase in enhanced solubility and bioavailability of naringenin [38]. Hence, the use of naringenin liposomal nanoformulations regarding to the useful effects of naringenin as a retinoprotective agent and liposomes as ocular drug delivery nanoparticles can have a beneficial function in the treatment of retinopathy.

Our results also demonstrated an extensive angiogenesis in the retina of placebo group compared to control group. However, treatment with bevacizumab and three doses of NLF especially 800 µg/mL could reverse retinal damage caused by injection of α-AAA. Researchers have shown anti-angiogenesis effect of naringenin for treatment of malignant melanoma [53]. It has also shown that naringenin could inhibit corneal neovascularization by anti-inflammatory and antioxidant mechanisms.

In the bevacizumab group, the retinal stroma had a limited number of vessels and was very similar to the control group. It was also seen that the number of blood vessels reduced in the NLF treated group with a concentration of 200 µg/mL, but a number of dilated vessels were still observed. Furthermore, the number of blood vessels also reduced in the NLF treated group with a concentration of 500 µg/mL and a much smaller number of dilated blood vessels were seen. In the NLF treated group with a concentration of 800 µg/mL, the microscopic structure showed a high improvement and was very similar to the bevacizumab and control groups.

The strength of the current study can be seen as its novelty. This means that the preparation of liposomal nanoparticles from naringenin and its effect on retinopathy has been done for the first time. Also, choosing the rabbit model as a standard model for investigating neovascularization was another strength of this study. However, limitations such as not investigating the cytotoxicity of prepared nanoparticles are also observed in this study.

Preparation of bioactive nanoformulation of naringenin

loaded into liposome was successfully carried out. SEM imaging confirmed successful formulation of NFL. The particle size, zeta potential and encapsulation efficiency of NFL revealed a good value for a lipophilic compound such as naringenin. The release and stability of NFL was reasonable. NFL exhibited ameliorative effects against neovascularization caused by α-AAA through reduced clinical score of neovascularization. Taken together, the present study demonstrated that the NFL suggested a capable nanoformulation with the peaked attenuating effect against neovascularization. Finally, the findings revealed that NFL could be proposed as a potential anti-neovascularization agent in retinopathy.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (F. Bagheri). The data are not publicly available due to privacy or ethical restrictions.

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Ethical Approval

The Animal Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS) approved all of the experimental protocols (IR.AJUMS.ABHC.REC. 1399.064).

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Competing of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Author Contributions

AS, BSH and MF conceived and supervised this study. BSH, SHM and MF completed the main experimental content. AS, AR and FB collected and analyzed data. FB and AS wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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